Report No:

Title: **Activated Sludge Respiration Inhibition Test of**

Study No:

External Testing Facility No:

Test Article:

Study Director:

Sponsor:

Sponsor Representative:

Testing Facility:

Study Completion Date: **November 10, 2000**

SECURITY STATEMENT:

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ABSTRACT

The primary objective of this study was to evaluate the test article, for its potential inhibitory effects on microbial respiration in an activated sludge suspension. The respiration rate of an activated sludge inoculum in a synthetic sewage suspension, after aerating for 3 hours in the presence of Int., was compared to the respiration rate of an activated sludge inoculum in a synthetic sewage suspension to which no test article was added.

Two control flasks containing an activated sludge and synthetic feed mixture with no test article added were also tested. The two control respiration rates were not within 15% of each other as required by protocol but were within 20%.

A reference article, 3,5-dichlorophenol, a known microbial inhibitor was tested to verify normal sensitivity of the microbial population. Reference article concentrations of 3.2, 10, and 32 mg/L resulted in percent inhibition values of 33, 30, and 81%, respectively, during the test. An EC_{50} value of approximately 15 mg/L was determined for the reference article and is within the acceptable range of 5 to 30 mg/L as specified in the OECD Guidelines, Section 209.

An abiotic control was dosed at approximately 100 mg/L during the test and was used to measure chemical oxygen uptake. No inoculum was added to this flask. No oxygen uptake was measured in the abiotic control.

The preliminary test was conducted at concentrations of approximately 1, 5, 25, 50, and 100 mg/L. Inhibition levels of 10 and 13% were observed at concentrations of 50 and 100 mg/L, respectively. Since the percent inhibition at the highest concentration tested is <50%, an EC_{50} (median effective concentration, the concentration at which 50% inhibition was observed) value for could not be determined and a definitive test was not performed.

GLP COMPLIANCE STATEMENT

This is the GLP Compliance Statement for Study No. and Study No. entitled "Activated Sludge Respiration Inhibition Test of for

The study sponsor will be responsible for characterization of the test article. The GLP characterization data for this test article will be reported in a report. The data for the test article characterization will be archived at No retention sample of test article was archived. The certificate of analysis for the reference substance was provided by the supplier. The study director for this test cannot testify to the GLP compliance of these data.

study director for the above test confirms that the study was conducted in compliance with the OECD Principles of Good Laboratory Practice (1) and the U.S. EPA, Toxic Substances Control Act: Good Laboratory Practice Standards (2), with the following exception:

> The characterization of the reference article was not conducted in compliance with Good Laboratory Practice Standards.

This compliance issue did not affect the integrity of the study.

A copy of the final report will be provided to All original raw data and/or certified copies of certain raw data records in support of this report, along with facility records, the original final report, and the original protocol and alterations will be retained at

Study No. -
External No. -

QUALITY ASSURANCE STATEMENT

This is the Quality Assurance Statement for Study No. and entitled "Activated Sludge Respiration Inhibition Test of for

This report was reviewed by Quality Assurance Unit. The following inspections were conducted on this study:

Date of Inspection	Phase Inspected	Date Reported to Study Director	Date Reported to Management
13 June 2000	Protocol	21 June 2000	21 June 2000
6 July 2000	Dosing	6 July 2000	10 July 2000
10 August 2000	Draft Report/ Raw Data	10 August 2000	30 August 2000
2 November 2000	Final Report	2 November 2000	3 November 2000

These audits indicate that the report submitted is an accurate reflection of the study as it was conducted by ABC Laboratories, Inc., and that the protocol and applicable SOPs were followed.

10 Nov 00
Date

Quality Assurance Officer I

APPROVAL SIGNATURES

This report consists of pages 1 through 23 including Tables 1 through 4.

Name: Date 16 Nov 2000

Degree: Bachelor of Science

Title: Scientist, Environmental Fate and Effects

Study Director

Name: Date 10 Nov 00

Degree: Master of Science

Title: Manager, Chemical Services, Environmental Fate and Effects

Performing Laboratories' Manager

Name: Date Nov. 7, 2000

Degree: Bachelor of Science

Title: Aquatic Toxicologist

Sponsor Representative

STUDY INFORMATION

Study Initiation Date:	21 June 2000
Experimental Start Date:	6 July 2000
Experimental Termination Date:	10 July 2000
Study Completion Date:	10 November 2000
Study Director:	
Sponsor:	
Sponsor Representative:	
Study Personnel:	

I. INTRODUCTION

contracted to conduct an activated sludge respiration inhibition test of The purpose of this test was to provide a screening method to identify substances which may adversely affect aerobic microbial treatment plants and to determine non-inhibitory concentrations of a test article to be used in biodegradation tests. The study was conducted as described in Protocol titled "Activated Sludge Respiration Inhibition Test of which was patterned after OECD Guideline 209 (3).

II. OBJECTIVE

The purpose of the test was to estimate the effect of to microorganisms by measuring the respiration rate under defined conditions in the presence of varying concentrations of the test article. An EC_{50} value may then be calculated. The EC_{50} is the concentration of the test article at which the respiration rate is 50% of that shown by the control under conditions described in the guideline.

III. TEST AND REFERENCE ARTICLE INFORMATION

A. Test Article

was received from on June 27, 2000. The test article was assigned reference number The test article was stored at room temperature when not in use. The following information was also provided by the sponsor regarding the test article:

Identity:	
Batch Number:	0000439367
Container Accession Number:	
Physical Description:	Clear Liquid
Handling Precautions:	Normal laboratory precautions
Solubility:	Readily soluble
Storage Conditions:	Room temperature
Purity:	Not applicable
Stability:	Stable at ambient temperatures in closed containers
Expiration Date:	December 17, 2000

B. Reference Article

The reference article, 3,5-dichlorophenol, was received from (Milwaukee, WI) on March 13, 1997. The reference article was assigned The following information was taken from the certificate of analysis and MSDS which were provided by the supplier regarding the reference article. Copies of these documents are maintained in the study files.

Identity:	3,5-dichlorophenol
Lot Number:	09615KG
CAS number:	
Empirical formula:	
Manufacturer:	
Physical Description:	White crystalline powder
Solubility:	Not given
Storage Conditions:	Store in freezer
Purity:	97.9% (by GC analysis)
Stability:	Incompatible with acid chlorides, acid anhydrides, and oxidizing agents
Expiration Date:	August 22, 2000

IV. ROUTE OF EXPOSURE

was dosed into the test flasks in a reagent water solution at the five different test article concentrations. This route of exposure was chosen to meet the requirements of OECD Guideline 209 (3). The duration of the exposure was 3 hours.

V. TEST WATER

A. Well Water

well water (non-chlorinated) was used for all inoculum preparation procedures, synthetic sewage feed preparation, and test flask preparations. The water was used directly from the faucet in the laboratory with no additional preparation.

B. Reagent Water

reagent water was purified, deionized, and filtered using a Millipore Milli-Q Water Purification System. The filtrate was >10 megohms•cm in resistivity, which is equivalent to or better than the ASTM Type II water resistivity requirement.

VI. TEST SYSTEM

The microbial inoculum used in the test was activated sludge collected on July 5, 2000, from aeration basin #1 at the Columbia Wastewater Treatment Plant in Columbia, Missouri. Approximately 11 liters of activated sludge were collected. Upon arrival at the sludge was centrifuged and washed with well water. The washed sludge was aerated and stirred for one day until addition to the study test mediums. Synthetic sewage feed was added daily to the sludge at a concentration of approximately 50 mL per liter of sewage. Two hundred milliliters of the activated sludge were used as the inoculum for each contact flask.

VII. EXPERIMENTAL DESIGN

A. Exposure System

Each test system consisted of a 1000-mL glass flask filled with a mixture of well water, inoculum, feed solution and the appropriate amount of test or reference article. The contact flasks were kept in a temperature-controlled environmental chamber (Norlake® Scientific, Hudson, WI). The contact flasks were labeled with treatment type ("Test", "Ref.", "Control", or "Abiotic"), concentration (mg/L for test or reference flasks), date, operator initials, and replicate number (for the duplicate control flasks). The beginning of the 3-hour contact period was marked by the addition of 200 mL of the activated sludge solution and either the test or reference article to each contact flask; the control flasks received no test or reference article and the abiotic control received no activated sludge. The final volume in each flask was 500 mL. Each contact flask was aerated for ~3 hours at 20 ± 2°C. The contact flask preparations were staggered at approximately 15-minute intervals to allow for oxygen consumption analysis and pH determination (Accumet model 50, Arvada, CO) at the end of each 3-hour contact period. The oxygen consumption rate was measured using a YSI model 58 dissolved oxygen meter (Yellow Springs, OH) connected to a chart recorder (Cole-Parmer model 2020, Chicago, IL).

B. Randomization

The order of preparation of the test systems in the environmental chamber was randomized using a SAS randomization computer program (4). The control flasks were not randomized but were prepared at the beginning and end of the series of test flasks.

C. Activated Sludge Preparation

Twelve 250-mL centrifuge bottles were filled with activated sludge and centrifuged at ~3000 rpm for ~5 minutes using an IEC CR-6000 centrifuge. The supernatant was discarded, more activated sludge was added to the bottles, and these bottles were centrifuged again. The supernatant was then discarded. The activated sludge was rinsed by adding well water, shaking, and then re-centrifuging. The supernatant was discarded and the activated sludge was rinsed twice more in the same manner. The final supernatant was discarded. A suspension of the sludge pellets was prepared by adding 200 mL of well water to each bottle. The contents of the bottles were combined in a 5-L carboy.

The combined sludge volume of 2500 mL was placed in an environmental chamber at 20 ± 2°C and aerated using compressed air. Synthetic sewage feed was added to the sludge at approximately 50 mL/L to sustain it overnight.

D. Feed Solution

Synthetic sewage feed was prepared as outlined in Table 1.

E. Compressed Air

The microbial inoculum and solutions in contact flasks were aerated with oil-free compressed air. The compressed air was breathable air composed of oxygen (19.5 to 23.5 %) with the balance as nitrogen.

F. Suspended Solids Concentration Determination

The suspended solids concentration in the activated sludge solution was determined by filtering five 1-mL aliquots of sludge through pre-weighed glass-fiber filter pads, followed by drying in an oven at approximately 100°C. The increase in weight of the filter pads was used to determine the

suspended solids level. The suspended solids concentration of the final activated sludge solution used for the test was 3.8 g/L.

G. Dose Groups — Preliminary Test

1. Test Article Administration

A stock solution of the test article was prepared by weighing 0.2514 g of and diluting to a final volume of 50 mL using reagent water. The concentration of was 5.03×10^3 mg/L. This stock solution was used to add test article to the appropriate flasks. The test article stock solution was stored refrigerated.

2. Reference Article Administration

A stock solution of the reference article was prepared by weighing 0.5155 g of 3,5-dichlorophenol, correcting for purity of 97.9 %, and diluting in 10 mL of 1N NaOH. This solution was diluted to 30 mL using reagent water; and 1N H_2SO_4 was added until the 3,5-dichlorophenol reached the point of incipient precipitation. The solution was then diluted to a final volume of 1000 mL using reagent water. The final concentration of 3,5-dichlorophenol was 505 mg/L. This stock solution was used to add reference article to the appropriate flasks. The reference article stock solution was stored refrigerated.

3. Contact Flasks Preparation

The preparation of the contact flasks for the preliminary test is summarized in Table 2. Each flask contained a Teflon-coated magnetic stir bar and was placed on an insulated magnetic stir plate. Control flasks contained the activated sludge inoculum, well water, and synthetic sewage feed but no test or reference article. Test article flasks, containing the activated sludge inoculum, well water, and synthetic sewage feed, were dosed with the test article solution at concentrations of 1, 5, 25, 50, and 100 mg/L. Reference article flasks, containing the activated sludge inoculum, well water, and synthetic sewage feed, were dosed with the reference article solution at concentrations of 3.2, 10 and 32 mg/L. The abiotic control flask contained well water, synthetic sewage feed, and test article (at a concentration of 100 mg/L) but no activated sludge

inoculum. The flasks were dosed in the following order: Control 1, Reference 3.2 mg/L, Reference 32 mg/L, Test 50 mg/L, Test 100 mg/L, Reference 10 mg/L, Test 25 mg/L, Abiotic Control, Test 1 mg/L, Test 5 mg/L, and Control 2 using the randomization program. The contents of each flask were stirred and aerated at 1000 mL/min throughout the ~3−hour contact period. After the ~3− hour contact period, the oxygen consumption rate in each flask was determined.

Based on the results from the preliminary test, a definitive test was not conducted.

H. Parameters Measured

After a ~3-hour contact period, the following procedure was performed on each flask:

1. Aeration was stopped.

2. The activated sludge suspension (~300 mL) from the contact flask was transferred into a BOD bottle containing a magnetic stir bar. The probe was then inserted into the bottle leaving no headspace. The solution in the BOD bottle was stirred using an insulated magnetic stir plate and stir bar.

3. The dissolved oxygen concentration of the activated sludge suspension was measured and recorded on a chart recorder for four to ten minutes during which time a linear response was observed in the depletion of oxygen.

4. The pH of the remainder of the flask contents was determined.

5. The oxygen consumption rate was determined from the slope of the line on the chart recorder trace, which was a plot of dissolved oxygen concentration (mg O_2/L) versus time (minutes).

I. Temperature Recordings

The test systems were incubated in an environmental control chamber for which the temperature was recorded throughout the test using a circular chart recorder. The temperature range recorded during the study was 19 to 21°C.

J. Calculations

1. Oxygen Consumption Rate

The oxygen consumption rate (respiration rate) was plotted as mg O_2/L per minute. The oxygen consumption rates were multiplied by 60 minutes to express results as mg O_2/L-hr.

2. Percent Inhibition

The percent inhibition for each concentration of test or reference article was calculated according to the following equation:

$$\% \text{ Inhibition} = \left[1 - \frac{R_S}{R_C}\right] \times 100$$

where:

R_S = oxygen consumption rate (mg O_2 L^{-1} hr^{-1}) at a given concentration of test or reference article

R_C = mean oxygen consumption rate for controls (mg O_2 L^{-1} hr^{-1}), rounded to nearest whole number

K. Statistical Methods

The median effective concentration (EC_{50}) for the reference article was statistically calculated using an EC_{50} SAS Program (4). The program calculates the EC_{50} statistic and its 95% confidence limits using the Spearman-Karber method (5). The variables were calculated concentrations and percent inhibition at the corresponding concentration.

L. Deviations

The respiration rates of the two controls were not within 15% of each other as required by protocol but were within 20%. There is no adverse impact to this study since the EC_{50} value for the reference article (15 mg/L) is within the acceptable range of 5 to 30 mg/L as specified in the

OECD Guidelines, Section 209 and no significant respiration inhibition was observed for the test article.

VIII. RESULTS AND DISCUSSION

The preliminary test was performed without replication at nominal concentrations of approximately 1, 5, 25, 50, and 100 mg/L of and 3.2, 10, and 32 mg/L of reference article, 3,5-dichlorophenol. The preliminary test was also performed on duplicate controls and a single abiotic control. A mixture of well water, inoculum, and feed solution was exposed to each concentration of and/or reference article. Based on the results from the preliminary test, no definitive test was conducted.

A. Biological Results

1. Control

The results of this test showed that the individual respiration rates of the two control flasks were not within 15% of the mean respiration rate as required by protocol but were within 20% (Table 3).

2. Test Article

In the preliminary test, 10 and 13% inhibition were observed at concentrations of 50 and 100 mg/L, respectively (Table 3). Since the percent inhibition at the highest concentration tested is <50%, an EC_{50} (median effective concentration, the concentration at which 50% inhibition was observed) value for could not be determined, a definitive test was not performed.

3. Reference Article

The three reference flasks exhibited percent inhibition values of 33, 30, and 81% at 3,5-dichlorophenol concentrations of 3.2, 10, and 32 mg/L, respectively (Table 3). An EC_{50} value of approximately 15 mg/L with 95% confidence limits of 12 and 19 mg/L was calculated for 3,5-dichlorophenol using the Spearman-Karber method. This EC_{50} was within the 5 to 30 mg/L range as required by the OECD Guidelines Section 209 (3).

B. Water Chemistry

The pH values for the study flasks taken after the 3-hour contact period ranged from 8.34 to 8.93 (Table 4).

IX. CONCLUSION

An EC_{50} value for could not be determined since the percent inhibition at the highest concentration tested is <50%. The EC_{50} value for the reference article, 3,5-dichlorophenol, was determined to be 15 mg/L and is within the OECD 209 guideline range of 5 to 30 mg/L.

X. ARCHIVES

Data for the test article characterization was archived at Data for the reference article characterization were archived at Additionally, instrument logbooks detailing calibration and maintenance, facility records, the original final report, all original raw data and/or certified copies for certain raw data and the original protocol and alterations were archived at A copy of the report, protocol, protocol alterations, and all supporting raw data were provided to

REFERENCES

(1) Organization for Economic Cooperation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97) 186/Final].

(2) U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act (TSCA). Good Laboratory Practice Standards. Final Rule (40 CFR, Part 792), EPA Washington, D.C.

(3) Organization for Economic Cooperation and Development, 1993. OECD Guidelines for Testing of Chemicals. Section 209, Activated Sludge, Respiration Inhibition Test, Adopted April 4, 1984, OECD, Paris, France.

(4) The SAS System for Windows, Release 6.12. Copyright 1989-1996 by SAS Institute, Inc., Cary, NC.

(5) Hamilton, M.A., R.C. Russo, And R.V. Thurston. 1977. Trimmed Spearman-Karber Method for Estimating Lethal Concentrations in Toxicity Bioassays. *Environ. Sci. Technol.* 11(7):714-719. Correction 12(4):417. 1978.

TABLE 1. Constituents of the Synthetic Sewage Feed Solution

Synthetic sewage feed was made by dissolving the following nominal amounts of substances per 1 L of well water.

Peptone (Difco Laboratories, Detroit, MI)	16 g
Beef Extract (Difco Laboratories, Detroit, MI)	11 g
Urea (Fisher Chemical, Fair Lawn, NJ)	3 g
NaCl (Sigma Chemical Co., St. Louis, MO)	0.7 g
$CaCl_2 \cdot 2H_2O$ (Fisher Chemical, Fair Lawn, NJ)	0.4 g
$MgSO_4 \cdot 7H_2O$ (Sigma Chemical Co., St. Louis, MO)	0.2 g
K_2HPO_4 (Fisher Chemical, Fair Lawn, NJ)	2.8 g

TABLE 2. Contact Flask Treatments

Flask	Test Article Solution (mL)[a]	Reference Article Solution (mL)[b]	Well Water (mL)	Synthetic Feed Stock (mL)	Activated Sludge Culture (mL)
Control 1	-	-	284.0	16	200
Control 2	-	-	284.0	16	200
Test 1 mg/L	0.1	-	283.9	16	200
Test 5 mg/L	0.5	-	283.5	16	200
Test 25 mg/L	2.5	-	281.5	16	200
Test 50 mg/L	5.0	-	279.0	16	200
Test 100 mg/L	10	-	274.0	16	200
Abiotic Control	10	-	474.0	16	-[c]
Reference 3.2 mg/L	-	3.2	280.8	16	200
Reference 10 mg/L	-	10	274.0	16	200
Reference 32 mg/L	-	32	252.0	16	200

Note: The final volume in each flask was 500 mL.
[a]Concentration of test article solution was 5.03×10^3 mg/L.
[b]Concentration of reference article solution was 505 mg/L.
[c]The abiotic control was dosed at a test article concentration of approximately 100 mg/L and was not inoculated with the activated sludge culture.

TABLE 3. Respiration Rates and Percent Inhibition Values for and 3,5-Dichlorophenol

Flask	Respiration Rate ($mg\ O_2L^{-1}\ hr^{-1}$) (R_s)
Control 1 (R_{C1})	36
Control 2 (R_{C2})	44
Mean ± 7.5%[a]	40 ± 3.0
15% Range of Mean[a] (Mean ± 7.5%)	37 – 43

	Respiration Rate ($mg\ O_2L^{-1}\ hr^{-1}$) (R_s)	% Inhibition[b]
1 mg/L	49	no inhibition
5 mg/L	47	no inhibition
25 mg/L	41	no inhibition
50 mg/L	36	10
100 mg/L	35	13
Abiotic Control (100 mg/L)	0.0	N/A

3,5-Dichlorophenol	Respiration Rate ($mg\ O_2L^{-1}\ hr^{-1}$) (R_s)	% Inhibition[b]
3.2 mg/L	27	33
10 mg/L	28	30
32 mg/L	7.5	81

[a] Calculation of 7.5% of mean and 15% range of mean is based on the unrounded mean before rounding the final value.

[b] Refer to section VII-J-2 for percent inhibition calculations.

TABLE 4. pH Value for Each Contact Flask

Treatment Flask	pH[a]
Control 1	8.35
Control 2	8.34
1 mg/L Test	8.34
5 mg/L Test	8.42
25 mg/L Test	8.46
50 mg/L Test	8.46
100 mg/L Test	8.53
Abiotic Control	8.93
3.2 mg/L Ref	8.56
10 mg/L Ref	8.60
32 mg/L Ref	8.59

Note: The pH of the sludge culture immediately preceding study initiation was 7.58.

[a]pH determined after the 3-hour contact period.